

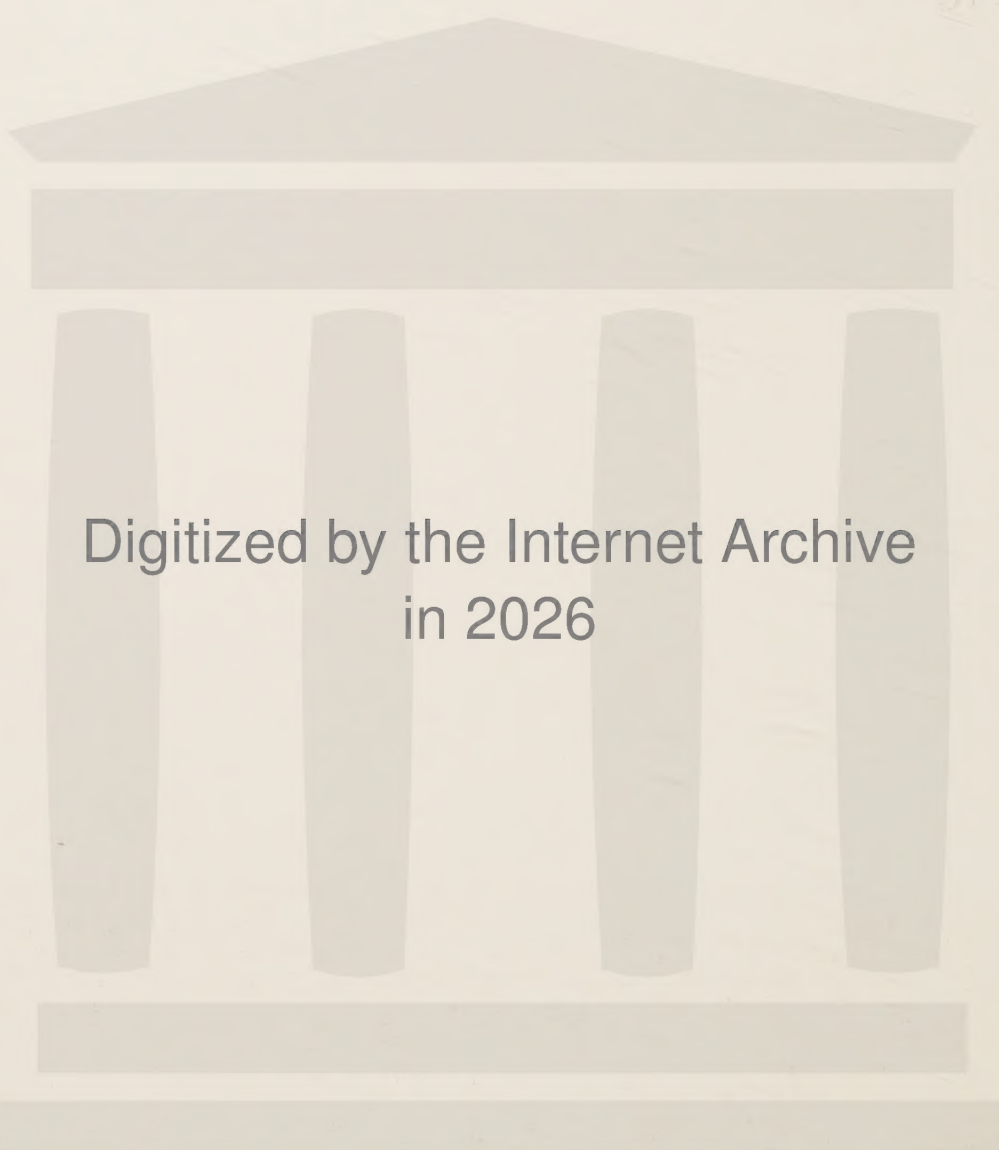
THYMINE 7-HYDROXYLASE — A 2-OXOGLUTARATE-DEPENDENT
DIOXYGENASE

Elisabeth Holme Markstedt

1982



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AKADEMISK AVHANDLING

som, för avläggande av medicine doktorsexamen vid Göteborgs Universitet, kommer att offentligen försvaras i föreläsningssal F3, Sahlgrenska sjukhuset fredagen den 2 april 1982, kl. 9.15

Elisabeth Holme Markstedt

The present thesis is based on the following publications:

- I Holme, E., Lindstedt, G., Lindstedt, S., and Tofft, M. 7-Hydroxylation of thymine in a neurospora strain coupled to oxidative decarboxylation of 2-ketoglutarate. Biochim. Biophys. Acta **212**, 50-57 (1970).
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- III Holme, E. A kinetic study of thymine 7-hydroxylase from Neurospora crassa. Biochemistry **14**, 4999-5003 (1975).
- IV Holme, E., and Lindstedt, S. On the partial reaction of thymine 7-hydroxylase in the presence of 5-fluorouracil. Biochim. Biophys. Acta. In press.
- V Holme, E. Determination of intra- and intermolecular tritium isotope effects in the reaction of thymine 7-hydroxylase. FEBS Lett. Submitted for publication.

ABSTRACT

Holme Markstedt, E. Thymine 7-hydroxylase — a 2-oxoglutarate-dependent dioxygenase. 1-36. Department of Clinical Chemistry, University of Gothenburg, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden.

The reaction catalyzed by thymine 7-hydroxylase from Neurospora crassa has been investigated in an attempt to improve the understanding of 2-oxoglutarate-dependent hydroxylase reactions.

Thymine 7-hydroxylase catalyzes the oxidation of thymine to 5-carboxyuracil in three consecutive hydroxylation reactions coupled to oxidative decarboxylation of 2-oxoglutarate. The dioxygenase character of the reaction has been settled by ^{18}O experiments. One oxygen atom from dioxygen is incorporated into each of the products formed i.e. both into the hydroxylated products and into succinate. The steady state kinetics of the enzyme were in accordance with a sequential ordered mechanism, where 2-oxoglutarate, thymine, and O_2 bind to the enzyme in that order. 2-Oxoglutarate decarboxylation is uncoupled from hydroxylation in the presence of certain substrate analogues. The uncoupled reaction in the presence of 5-fluorouracil has been investigated. The steady state kinetics of the uncoupled reaction were found to be of the same kind as in the complete reaction. The demand for a reductant in the uncoupled reaction was greater than in the complete reaction. The intra- and intermolecular isotope effects of thymine hydroxylation have been determined. No intermolecular isotope effect was found even at reduced oxygen tension. The intramolecular $k_{\text{H}}/k_{\text{T}}$ was found to be about 6.5. A tentative reaction mechanism is proposed based on the experimental findings on thymine 7-hydroxylase together with findings on other 2-oxoglutarate-dependent dioxygenases as well as other both enzymatic and nonenzymatic hydroxylation reactions. A primary attack of the iron-bound dioxygen on the carbonyl carbon of 2-oxoglutarate to form a peroxy acid intermediate is envisaged. A heterolytic cleavage of the oxygen-oxygen bond then preceeds the formation of a Fe(IV)=O complex, which is the actual hydroxylating agent.

Key words: Thymine 7-hydroxylase, 2-oxoglutarate, dioxygenase, ^{18}O , steady state kinetics, uncoupling, tritium isotope effects.

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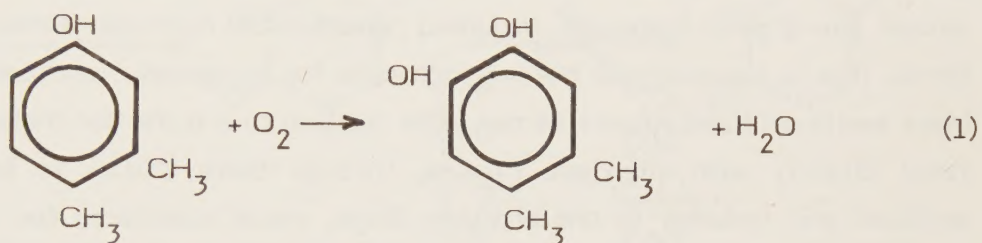
These studies were supported by grants from the Swedish Medical Research Council (13X-585) and the Medical Faculty, University of Gothenburg.

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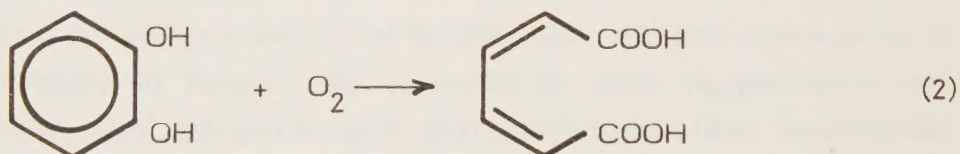
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INTRODUCTION

Evidence for enzyme-catalyzed insertion of molecular oxygen into organic molecules was presented in 1955. In experiments performed in an atmosphere enriched with the heavy oxygen isotope, ^{18}O , Mason et al. (1) could demonstrate that one atom of oxygen from atmospheric oxygen was inserted into 3,4-dimethylphenol in the reaction catalyzed by phenolase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1)(Eq 1).



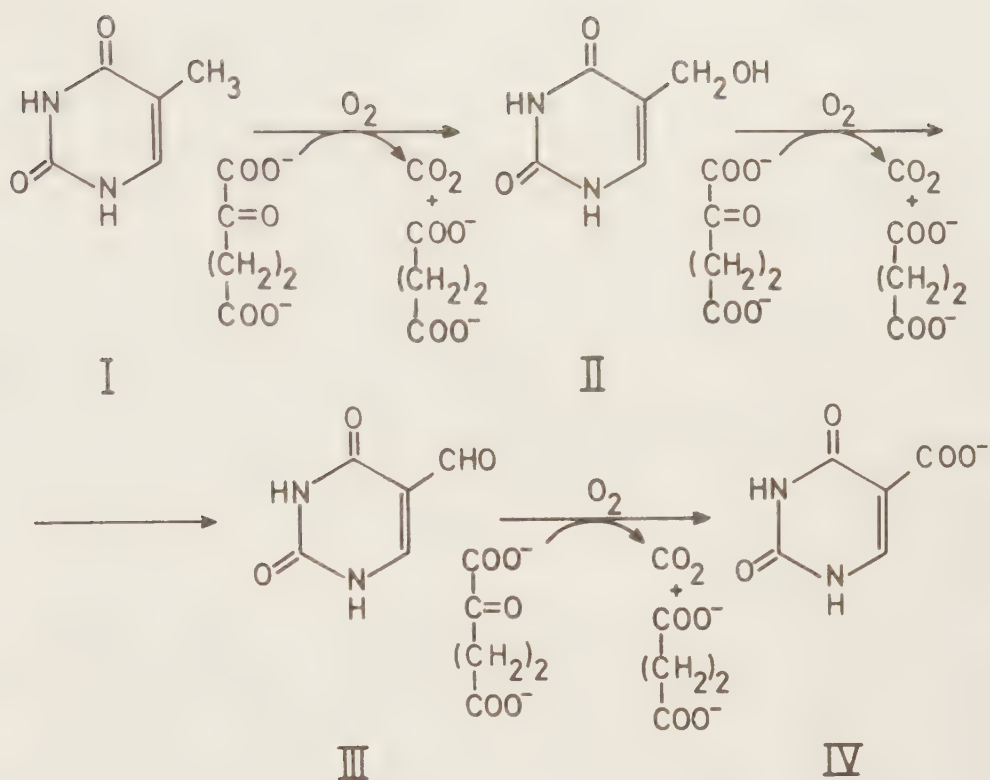
At the same time, in similar experiments, Hayaishi et al. (2) showed that two atoms of molecular oxygen were incorporated into catechol in the reaction catalyzed by pyrocatechase (catechol: oxygen 1,2-oxidoreductase (decyclizing) EC 1.13.11.1) (Eq 2).



The members of the new class of enzymes were called oxygenases after a proposition of Hayaishi et al. in 1956 (3). The early findings of oxygenase chemistry were summarized by Hayaishi in 1962 (4). Since then over one hundred oxygenase reactions have been identified (5). The occurrence of these enzymes is universal and of great importance in the metabolism of both endogenous and exogenous substances.

Oxygen in its ground state is a triplet with two unpaired electrons. Organic molecules are singlets. Reaction between a triplet molecule and a singlet molecule to form all-singlet products is spin forbidden and the kinetic barrier to reaction is large. The challenge of oxygenase chemistry since 1955 has been to identify the "active oxygen" and to clarify the mechanism for controlled oxidation at conditions compatible with life. A key role in oxygen activation is played by transition metals, mainly iron and copper, which through their ability to form oxygen complexes of different oxidation states, could convert dioxygen to an oxygen species suited for attack on almost any organic molecule, including nonactivated hydrogen-carbon bonds. This is, however, not the only principle for oxygenase reactions. More easily oxidized substrates can after ligation to a transition metal react directly with dioxygen. Flavins, through their ability to be oxidized and reduced in one-electron steps, could substitute for a transition metal in oxygenase reactions (Reviews are given in 6,7,8).

Oxygenases have been divided into dioxygenases and monooxygenases. In dioxygenase reactions both oxygen atoms are incorporated into the substrate. In monooxygenase reactions one oxygen atom is incorporated into the substrate whereas the other oxygen atom is reduced to water through electron transfer either from a cofactor or from the substrate itself. The 2-oxoglutarate-dependent dioxygenases offer a special problem with regard to classification. One oxygen atom is incorporated into the substrate, as in a monooxygenase reaction. The other oxygen atom is, however, not reduced to water but incorporated into a cosubstrate. The type of reaction catalyzed by these enzymes is illustrated in **Scheme I** with the reaction catalyzed by thymine 7-hydroxylase (thymine,2-oxoglutarate:oxygen oxidoreductase (7-hydroxylating), EC 1.14.11.6).



SCHEME I

I=Thymine, II=5-hydroxymethyluracil, III=5-formyluracil, and IV=5-carboxyuracil.

Thymine 7-hydroxylase is unique among the 2-oxoglutarate-dependent dioxygenases in catalyzing oxygenation of the formed alcohol to an aldehyde which is further hydroxylated to a carboxylic acid.

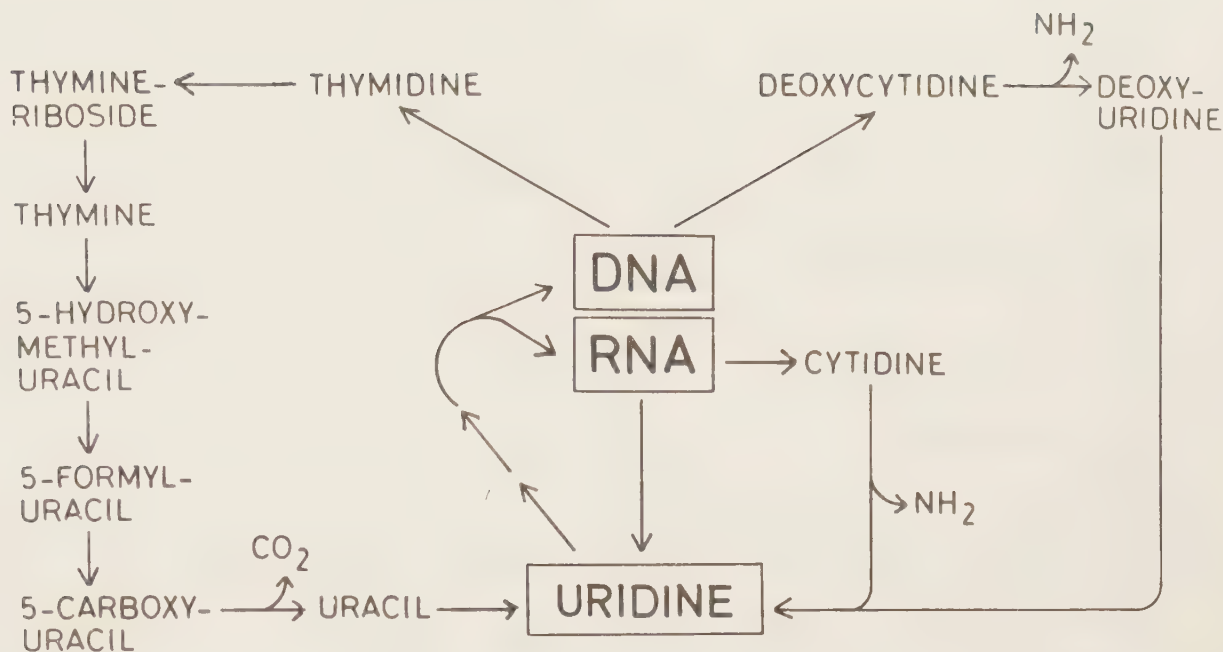
Sequential oxygenation of a methyl group in analogy to the reaction catalyzed by thymine 7-hydroxylase has been reported for kaurene in a reaction catalyzed by a NADPH-dependent particulate enzyme of *Echinocystis macrocarpa* (9).

A specific role for 2-oxoglutarate in oxygenase reactions was established in 1968 when it was shown that 2-oxoglutarate was converted to succinic acid in a 1:1 stoichiometry with hydroxylation of γ -butyrobetaine (10) and of prolyl residues of procollagen (11) in reactions catalyzed by γ -butyrobetaine hydroxylase (4-trimethylamino-butyrate, 2-oxoglutarate: oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1) and prolyl 4-hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate: oxygen oxidoreductase, EC 1.14.11.2) respectively.

2-Oxoglutarate-dependent dioxygenases have been found in both animal and plant tissues as well as in microorganisms. In addition to the

above mentioned enzymes this group now includes: lysyl 5-hydroxylase (peptidyllysine,2-oxoglutarate:oxygen oxidoreductase (5-hydroxylating), EC 1.14.11.4), prolyl 3-hydroxylase (EC 1.14.11.), thymidine 2'-hydroxylase (thymidine, 2-oxoglutarate:oxygen oxidoreductase (2'-hydroxylating), EC 1.14.11.3), and trimethyllysine 3-hydroxylase (1.14.11.) (Reviews are given in 6,7,12,13). These enzymes have thus been found in three metabolic pathways, but in each of these pathways more than one 2-oxoglutarate-dependent oxygenase is involved. Lysyl 5-hydroxylase and the two prolyl hydroxylases are involved in the conversion of procollagen to collagen; γ -butyrobetaine and trimethyllysine 3-hydroxylase are involved in carnitine synthesis; thymine 7-hydroxylase and thymidine 2'-hydroxylase are enzymes in a salvage pathway for pyrimidines.

In studies on the metabolism of thymine in rat liver slices, Fink et al. (14) could identify 5-hydroxymethyluracil and 5-carboxyuracil as oxidation products of thymine. This oxidative pathway for thymine has then mainly been studied in Neurospora crassa where a high activity of this enzyme system has been found (15,16,17,18). High activity has also been found in the related organism Rhodotorula glutinis (19). The pathway consists of consecutive hydroxylations of thymine to 5-carboxyuracil, which is then decarboxylated to form uracil (20). Uracil can be converted to uridyate and reenter the pathway for RNA and DNA synthesis (21,22) according to **Scheme II**. A reutilization pathway for cytidine is well established in mammalian tissue, where deamination of cytidine and deoxycytidine occurs (23,24,25). However, besides the early studies of Fink concerning oxidative metabolism of thymine in rat liver slices, there is only one report on mammalian thymine oxidation. It describes a severalfold increase in the conversion of thymine to 5-hydroxymethyluracil in rat leukemic cells compared to normal leukocytes (26). This indicates that salvage of thymine could be of biological importance in rapidly growing tissue. The other 2-oxoglutarate-dependent oxygenase involved in pyrimidine metabolism in Neurospora crassa, thymidine 2'-hydroxylase, which converts thymidine to thymine-riboside and deoxyuridine to uridine seems to catalyze the main metabolic pathway for thymidine and deoxycytidine in Neurospora, where low and variable activities of deoxypyrimidine nucleosidase has been found (27).



SCHEME II

PURIFICATION OF THYMINE 7-HYDROXYLASE

Several attempts to purify thymine 7-hydroxylase from *Neurospora crassa* by Abbott's group (28,29,30) and by us have not resulted in homogeneous enzyme preparations. Like the other 2-oxoglutarate-dependent dioxygenases this enzyme is rapidly inactivated at 4°C, and the enzyme concentration in the raw material is rather low. By using a mutant strain (uc-1) and by changes in growth conditions Abbott's group have succeeded to enrich thymine 7-hydroxylase in the raw material considerably (22), which seems to have improved the specific activity obtained (31).

We have used a purification procedure (Table I) which results in an enzyme preparation with a specific activity of about 15 $\mu\text{kat/g}$ (extrapolated $V_{\text{max}} = 26 \mu\text{kat/g}$). The purification is done in phosphate buffer at pH 6.5 with the addition of 0.1 M glycine, which stabilizes the enzyme (32). The first step is a chromatography on a hydroxylapatite column. In this step an activation of the enzyme occurs and the total recovery compared to the soluble fraction of the extract is about 200%.

Table I. Purification of thymine 7-hydroxylase from Neurospora crassa.

Enzyme fraction	Protein mg	Specific activity ₁ μkat g ⁻¹	Recovery %
Cell-free extract soluble fraction	2300	0.005	-
Eluate from hydroxylapatite column	137	0.20	100 ^a
Eluate from DEAE-cellulose column	36	0.68	89
Eluate from Sephadex G-100 column	0.3	15	16

^aThe total enzyme activity constantly increased in the first purification step, hence the total recovery is compared to the activity in this enzyme fraction.

The combined fractions with the enzyme activity from the hydroxylapatite column are put onto an anion-exchange chromatography column (Whatman DE 52) directly or after adjustment of the ionic strength by dilution. The addition of 0.1 mM EDTA from this stage on further stabilizes the enzyme. The ion-exchange chromatogram, is developed with a potassium chloride gradient. The eluate with the enzyme activity is concentrated by ultrafiltration. The enzyme preparation can then be kept at -70°C for months without any appreciable loss of enzyme activity. This enzyme preparation has been used for most mechanistic studies.

Before further purification by gel filtration another concentration step is included. This is achieved through precipitation with ammonium sulfate added in saturating amounts. The gel filtration of the redissolved precipitate is done on Sephadex G-100 superfine in two 100 cm columns coupled together (**Figure 1**). This enzyme preparation catalyzes the three consecutive 2-oxoglutarate-dependent hydroxylations of thymine to 5-carboxyuracil, it has also the ability to catalyze a pyrimidine-dependent decarboxylation of 2-oxoglutarate, which is not coupled to hydroxylation (33). The other 2-oxoglutarate-dependent hydroxylase involved in thymine metabolism in Neurospora crassa i.e. thymidine 2'-hydroxylase separates from thymine 7-hydroxylase in the ion-exchange chromatography step (32).

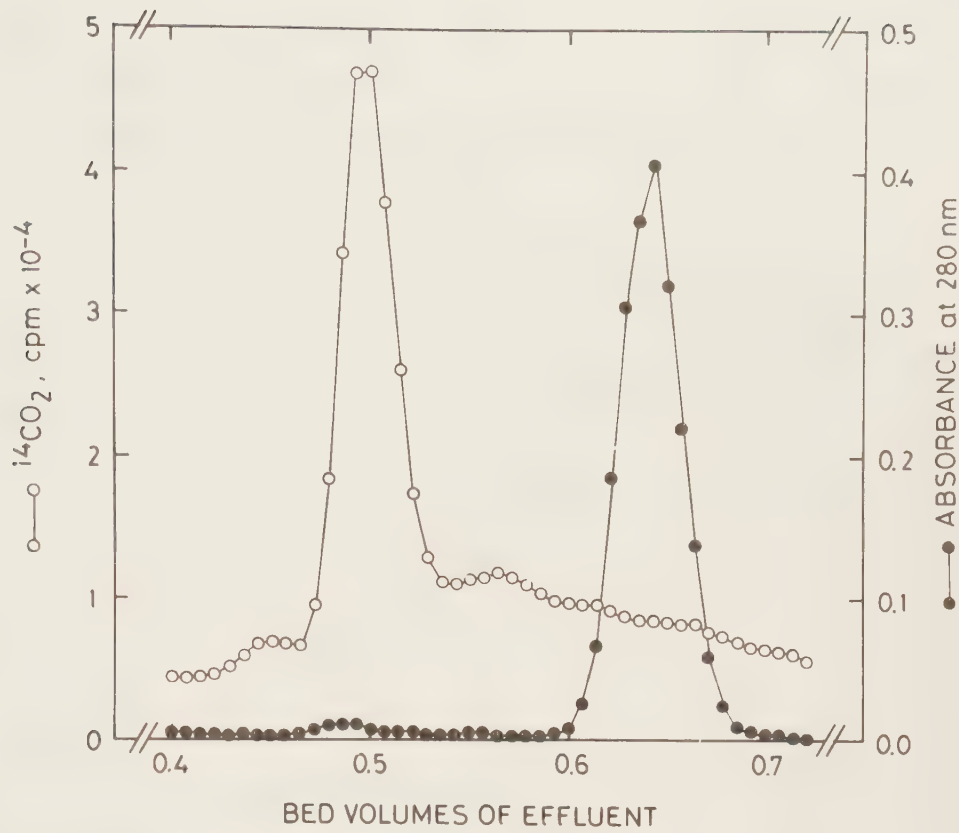


Figure 1. Gelfiltration of thymin 7-hydroxylase on Sephadex G-100 superfine in two 100 cm columns coupled together.

The molecular weight of thymine 7-hydroxylase is 41 000 as determined by gelfiltration and the isoelectric point is 4.9 (34). The purified enzyme preparations is totally dependent on addition of ferrous ion for activity.

MOLECULAR PROPERTIES OF 2-OXOACID-DEPENDENT DIOXYGENASES

Table II lists the molecular properties of the 2-oxoglutarate-dependent dioxygenases. Data on 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) are also included since there are good reasons to assume similar reaction mechanisms for this enzyme and the 2-oxoglutarate-dependent dioxygenases (35,36).

It is noteworthy that all the homogeneous 2-oxoglutarate-dependent oxygenases, if extensively studied, have been found to consist of two different subunits, in most cases of similar size.

Table II. Molecular properties of 2-oxoglutarate-dependent dioxygenases and 4-hydroxyphenylpyruvate dioxygenase^a

Enzyme	Enzyme source	Preparation	Molecular mass kdalton	Subunits	Iron content mol mol ⁻¹	Specific activity μkat g ⁻¹ mol s ⁻¹ mol ⁻¹	Molecular activity
γ-Butyrobetaine hydroxylase (37)	<u>Pseudomonas</u>	Homogeneous	95	α ₂ β	0.1	360 ^b	34
γ-Butyrobetaine hydroxylase (38)	Calf liver	"	80	2 ^c	- ^d	0.9	0.07
Trimethyllysine hydroxylase (39,40)	Rat liver and kidney	Mitochondria	-	-	- ^e	-	-
Trimethyllysine hydroxylase	<u>Neurospora</u>	Soluble fr.	-	-	- ^e	-	-
Prolyl 4-hydroxylase (42,43)	Chick embryo	Homogeneous	240	2α ₂ 2β	- ^d	25 ^b	6
Prolyl 4-hydroxylase (44)	Chick embryo	"	240	2α ₂ 2β	1	43 ^b	10
Prolyl 4-hydroxylase (45)	Human fetus	"	240	2α ₂ 2β	- ^e	23 ^b	6
Lysyl 5-hydroxylase (46)	Pig fetus	"	185	α ₂ β	- ^e	f ⁻	f ⁻
Lysyl 5-hydroxylase (47)	Chick embryo	"	200	2 ^c	- ^d	20 ^b	4
Prolyl 3-hydroxylase (48)	Chick embryo	Purified	160	-	- ^d	f ⁻	-
Prolyl 3-hydroxylase (49)	Rat kidney	15 000 x g sup	-	-	- ^e	-	-
Thymidine 2'-hydroxylase (34)	<u>Neurospora</u>	Purified	47	-	- ^d	2 ^g	-
Thymine 7-hydroxylase (see text)	<u>Neurospora</u>	Purified	41	-	- ^d	26 ^b	-
4-Hydroxyphenylpyruvate hydroxylase (50-52)	Human liver	Homogeneous	87	α ₂ β	0.4	67 ^b	6
4-Hydroxyphenylpyruvate hydroxylase(53)	<u>Pseudomonas</u>	"	150	4α	1	333 ^b	50
4-Hydroxyphenylpyruvate hydroxylase (54)	Chick liver	"	97	2 ^h	1	142	14

^a Table not complete with regard to enzyme source. ^b $V_{\max}$ values, either obtained through extrapolation or assayed at saturating substrate concentrations. ^c Equally sized subunits, not further characterized. ^d Not determined, low or no activity without added Fe²⁺. The references, with regard to Fe²⁺ dependence are given in "Ferrous ion and reduction". ^e Fe²⁺ dependence not specified. ^f Enzyme activity only given in arbitrary units. ^g Unpublished result from our laboratory. ^h Equal size, different charge.

The iron content of the purified enzymes is generally low and addition of iron is needed for full activity. Nietfeld and Kemp (44) have, however, isolated a homogeneous preparation of prolyl 4-hydroxylase which contains 1 mol Fe/mol of enzyme.

For this enzyme (44) as well as for the pseudomonad (55) and avian (54) 4-hydroxyphenylpyruvate dioxygenase an EPR signal at $g=4.3$, which is characteristic of ferric ion in a rhombic ligand field, has been reported. For the pseudomonad 4-hydroxyphenylpyruvate dioxygenase an absorbance maximum around 600 nm has been found. On reductive activation of the enzyme the color disappears - it has been ascribed to the Fe^{3+} content of the enzyme (55). No absorption in the visible region has been reported for the iron-containing prolyl 4-hydroxylase. No absorptions have been found that can be attributed to any prosthetic groups.

SUBSTRATES

2-Oxoglutarate

The stimulatory effect of 2-oxoglutarate in the reaction catalyzed by thymine 7-hydroxylase was found by Abbott's group in 1967 (16). 2-Oxoglutarate had been found to be stimulatory in several hydroxylase reactions viz. prolyl 4-hydroxylase (56,57) lysyl 5-hydroxylase (58,59) and γ -butyrobetaine hydroxylase of animal and bacterial origin (60,61). In the reactions catalyzed by γ -butyrobetaine hydroxylase and prolyl 4-hydroxylase it was soon shown that 2-oxoglutarate was not an unspecific stimulatory substance, but a substrate, which was converted to succinic acid in a 1:1 stoichiometry with hydroxylation.

The oxidative decarboxylation of 2-oxoglutarate in 1:1 stoichiometry with hydroxylation in the reaction catalyzed by thymine 7-hydroxylase was shown in **paper I**. The specificity for 2-oxoglutarate is high in this reaction as well as in other 2-oxoglutarate-dependent hydroxylations (62 and references therein) and no or negligible activity is obtained with other oxo acids.

Pyrimidine

It was early found that not only thymine but also its hydroxylated products 5-hydroxymethyluracil and formyluracil were substrates in the reaction catalyzed by thymine 7-hydroxylase (17,18). There is a tight coupling between hydroxylation of thymine and oxidative decarboxyla-

tion of 2-oxoglutarate and a stoichiometry of 1:1 has been found in all situations tested. Abbott's group has reported 1:1 stoichiometry for 5-hydroxymethyluracil and formyluracil hydroxylation (29), but we have generally had an overproduction of CO_2 from 2-oxoglutarate compared to formyluracil formation from 5-hydroxymethyluracil. Bankel *et al.* (32) showed that thymidine is not a substrate in the reaction. The substrate specificity was further investigated by Bankel *et al.* (63) who showed that pyrimidines with oxygen or sulfur substituents on carbon 2 and 4 as well as alkyl groups on carbons 1 and 5 were substrates when the enzyme activity was measured as the production of carbon dioxide from 2-oxoglutarate. 1-Methyluracil was also shown to be converted to uracil in the reaction. In view of the uncoupled reactions reported for thymine 7-hydroxylase (33,31, **paper IV**) it is of course not sufficient to measure 2-oxoglutarate decarboxylation to assign a substance a role as a substrate. The 1,5-alkylated uracils have however hydroxylatable groups and the activities found with these substances are of the same magnitude as that found with thymine. The activities found in the presence of pseudosubstrates are in a much lower range.

The reactions catalyzed by the other 2-oxoglutarate-dependent dioxygenases are stereospecific, which means that further hydroxylation of the same carbon can not be expected. One may, however, consider the possibility that hydroxylation could occur if the enantiomer of the product is used as a substrate. This type of reaction has been reported for dopamine β -hydroxylase (EC 1.14.17.1), which catalyzes hydroxylation of the product enantiomer S-octopamine (64). We have tested D-carnitine as a substrate in the reaction catalyzed by γ -butyrobetaine hydroxylase where L-carnitine is the normal product. D-Carnitine was not further hydroxylated but caused a substantial uncoupling of 2-oxoglutarate decarboxylation from hydroxylation. Although cis-4-hydroxyproline is incorporated into collagen in place of proline (65), there is no report on its reactivity towards prolyl 4-hydroxylase.

Oxygen

The need for oxygen in thymine hydroxylation was indicated by loss of activity when nitrogen or argon was substituted for air in the reaction (15, **paper I**). Oxygen was also required for hydroxylations of 5-hydroxymethyluracil (17) and 5-formyluracil (18). The crucial $^{18}\text{O}_2$ experiments needed to show the oxygenase character of the three consecutive hydroxylations of thymine were reported in **paper II**. It was shown that the formed 5-hydroxymethyluracil and 5-carboxyuracil had

incorporated one atom from molecular oxygen, and that one oxygen atom was incorporated into succinate formed in these reactions. No attempts were made to isolate formed 5-formyluracil since the carbonyl oxygen was expected to equilibrate with oxygen in water, the incorporation of ^{18}O into succinate was, however, shown. $^{18}\text{O}_2$ experiments have also been done with prolyl 4-hydroxylase, where incorporation of ^{18}O into 4-hydroxyproline and succinate was shown (66). The incorporation of ^{18}O from molecular oxygen into succinate had earlier been shown in the reaction catalyzed by γ -butyrobetaine hydroxylase (67).

FERROUS ION AND REDUCTION

In crude preparations of thymine 7-hydroxylase there is a variable degree of residual activity without any addition of ferrous ion to the medium. In the presence of iron chelators the enzyme is totally inactivated, but the enzyme activity is restored on addition of ferrous ion in excess of the chelator concentration. The specificity for Fe^{2+} is high and other transition-metal ions cannot replace Fe^{2+} (18). Fe^{2+} is lost during purification as is also the case with most other 2-oxoglutarate-dependent dioxygenases (37,68,69,70,71). Prolyl 4-hydroxylase has been purified to homogeneity with the retention of 1 mol Fe /mol enzyme (44), but even this preparation is further stimulated by the addition of Fe^{2+} . One explanation for this could be that the enzyme has more than one binding site for Fe^{2+} . This idea is substantiated by the results of Cumming *et al.* (72), who reported iron-binding experiments indicative of four iron binding sites per mole of enzyme. Another explanation is that enzyme Fe^{2+} is in equilibrium with Fe^{2+} in the solution which is in accordance with the steady state kinetics shown by Kivirikko *et al.* (70,71). Takeda *et al.* (73) have investigated the effect of several chelators on the activity of prolyl 4-hydroxylase and divided the chelators into three groups: group I which was inhibitory even when excess iron was added, group II where stimulation was found as soon as the iron concentration exceeded the chelator concentration, and group III which was stimulatory even when chelator concentration exceeded the concentration of Fe^{2+} . Nucleoside triphosphates were also included in group III. Takeda *et al.* (73) also showed that the effect of variation in Fe^{2+} concentration in the presence and absence of EDTA was quite different. In the absence of EDTA a slow rise in enzyme activity with increasing iron concentration was observed whereas in the presence of

EDTA a very rapid activation was observed as soon as the EDTA concentration was reached. The activity obtained at that iron concentration was twice as high as that in the absence of EDTA. We have observed similar effects for thymine 7-hydroxylase but the same maximal activity was reached in the absence of EDTA as in its presence, albeit at a higher iron concentration. A reductant is needed for full activity in the 2-oxoglutarate-dependent dioxygenase reactions; ascorbate has been found to be the most efficient (74 and references therein). A role for ascorbate would be to keep enzyme bound iron in the ferrous state (75). There are indications for the presence of SH-groups in the active site of prolyl 4-hydroxylase (76,12). It is known that the $S-Fe^{3+}$ complex in general is more stable than the $S-Fe^{2+}$ complex (77). The stimulatory effect of EDTA might be due to chelation and removal of Fe^{3+} from the enzyme which would facilitate exchange for Fe^{2+} , and this exchange may be more efficient than reduction of enzyme bound Fe^{3+} to Fe^{2+} . Takeda et al. did not investigate the chelator effects in relation to the need for ascorbate. Wondrach et al. (78), however, have shown that nucleoside triphosphate can substitute for ascorbate to a great extent in the thymine 7-hydroxylase reaction in Rhodotorula glutinis.

The need for a reductant in the 2-oxoglutarate-dependent oxygenases is not absolute. In our incubating system linear time-progress curves are obtained for about 2.5 min in the absence of ascorbate and the rate of product formation during that time is not significantly lower than in the presence of ascorbate. Other reductants like 2,6-dichloroindophenol and cysteine can substitute for ascorbate, although the efficiency varies with different enzyme preparations. Glutathione and dithiothreitol are generally less effective. Similar effects have been reported for other 2-oxoglutarate-dependent oxygenases (74 and references therein). For pure bacterial γ -butyrobetaine hydroxylase (79) and pure prolyl 4-hydroxylase (75) a more specific requirement for ascorbate has been found. The requirement, however, was not absolute and both enzymes could function at essentially maximal rate for a limited time. For prolyl 4-hydroxylase the reaction rate was unaffected by ascorbate for 15-30 reaction cycles, and no stoichiometric consumption of ascorbate was found when the reaction time was extended. For the iron-containing preparation of prolyl 4-hydroxylase ascorbate is needed for initiation of the reaction, which indicates that the enzyme-bound iron is in the ferric state (80). It was also shown that oxidative

inactivation of the enzyme in the presence of 2-oxoglutarate and oxygen was reversed by dithiothreitol but not by ascorbate, which is a weaker reductant. The inactivation by 2-oxoglutarate and oxygen may be due to an uncoupled reaction which leaves the enzyme in an oxidized form that cannot be reactivated by ascorbate. In the uncoupled reaction catalyzed by thymine 7-hydroxylase a considerably higher concentration of ascorbate is needed than in the complete reaction (**paper IV**). This indicates that the reductions are different in nature in the two reactions, and possibly that semidehydroascorbate, which in a redox couple with dehydroascorbate has a redox potential similar to dithiothreitol (80,81), is the actual reducing agent in the uncoupled reaction. Reduction could thus be needed to reverse the consequences of at least two different side reactions and also to keep free iron in solution in the ferrous state.

MECHANISM - EXPERIMENTAL EVIDENCE

Introduction

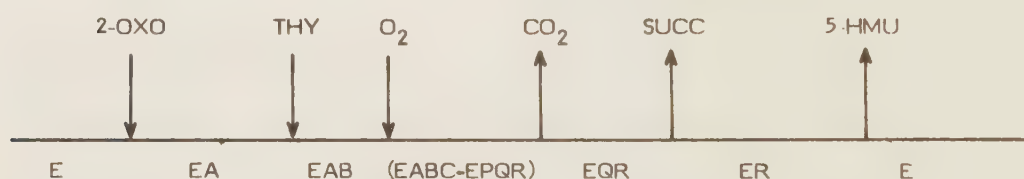
A reaction mechanism for the 2-oxoglutarate-dependent dioxygenases was proposed in 1968 (83). It was suggested that oxygen, activated by ferrous ion, initially attacked the substrate to be hydroxylated to form a peroxide, which would attack the carbonyl carbon of 2-oxoglutarate before breaking of the oxygen-oxygen bond.

The subsequent ^{18}O experiments confirmed the dioxygenase character of these reactions i.e. that both oxygen atoms are incorporated into organic substrates (**paper II**, 66,67).

This mechanism was criticized by Hamilton (36) who found it unlikely that a peroxide could be formed in a position which was completely unactivated. He proposed an initial formation of a peracid from 2-oxoglutarate. The formed monopersuccinic acid would then transfer an oxygen atom ("oxene") to the substrate. This hypothesis was tested by substituting monopersuccinic acid for 2-oxoglutarate in the thymine 7-hydroxylase reaction, monopersuccinic acid was also tested as an inhibitor in the reaction. The negative results obtained were, however, not easy to interpret since the peracid is rapidly converted to succinic acid in the presence of ferrous ion or ascorbate and at least ferrous ion is obligatory to get any measureable enzyme activity. Monopersuccinic acid has also been tested in the prolyl 4-hydroxylase and thymidine 2'-hydroxylase systems, without any effect (12).

Steady state kinetics

When the above mentioned reaction mechanisms were proposed nothing was known about the kinetic mechanism of the 2-oxoglutarate-dependent dioxygenases. There was only one report on prolyl 4-hydroxylase (84), but this study was done before 2-oxoglutarate had been recognized as an integral part of the reaction and was therefore of little value. Knowledge of the kinetic mechanism could give some direct information on the reaction mechanism and is essential for further mechanistic studies. Therefore a kinetic study of thymine 7-hydroxylase was initiated in spite of the methodological difficulties. The different initial velocity patterns and inhibition patterns expected for different kinetic ter reactant mechanisms had been described by Cleland (85,86,87,88,89), but experiments with ter reactant enzyme systems were few. The low capacity of the assay system especially when the oxygen concentration was varied and the fact that we did not have a continuous assay method, necessitated special care in setting up the experiments in order to get meaningful results. The different initial velocity plots in combination with inhibition studies eventually made up a pattern which was consistent with a sequential addition of substrates in an obligatory order according to **Scheme III** (paper III).



SCHEME III

The order of product release was, however, influenced by the presence of succinate which altered the order of release between succinate and 5-hydroxymethyluracil. The kinetic mechanism found, i.e. an ordered addition of substrate and binding of the organic substrates before oxygen, agrees with the findings for other dioxygenases (74), and for cytochrome P-450 enzymes (90).

Kivirikko has studied the steady state kinetics of prolyl 4-hydroxylase (70,91) and lysyl 5-hydroxylase (71,92) and concluded that the order of addition of substrates is 2-oxoglutarate, O₂, peptide substrate. The report on prolyl 4-hydroxylase is very hard to penetrate since the substrate concentrations given in the figures do not agree with the

calculated kinetic constants which are tabulated. The argument for placing O_2 as the second substrate is a parallel initial velocity plot where 2-oxoglutarate and peptide are varied at 1 mM O_2 concentration. The suggested order of addition of substrate may be the correct one at that O_2 concentration, but the corresponding initial velocity study performed in air, which would give an O_2 concentration about 5 times higher than the reported K_m for oxygen, does not agree with the proposed order of addition of substrates. The argument for placing O_2 as the second substrate in the reaction catalyzed by lysyl 5-hydroxylase has no experimental support in the report but is more of an assumption that the two enzymes act in the same way.

Since the kinetic mechanism is sequential i.e. all substrates are bound to the enzyme before any product is released it does not give any indications about which substrate is initially attacked by oxygen.

Uncoupling

Support for the mechanism proposed by Hamilton (36) was found when 2-oxoglutarate decarboxylation was detected in the absence of any added thymine in aged preparations of thymine 7-hydroxylase (93). These findings could not be reproduced with freshly prepared thymine 7-hydroxylase and have not been further investigated. The alternate situation i.e. hydroxylation of thymine or tritium release from [7- ^3H]thymine in the absence of 2-oxoglutarate has not been observed.

2-Oxoglutarate decarboxylation in the absence of peptide substrate had also been observed in the presence of prolyl 4-hydroxylase when substrate quantities of pure enzyme were used (12). It was shown in 1978 that this was a true uncoupling i.e. that no hydroxyproline was formed in any of the proteins in the reaction mixture (94). At that time it was also shown that uncoupling of 2-oxoglutarate decarboxylation from prolyl hydroxylation was greatly enhanced in the presence of competitive inhibitors to the polypeptid substrate (95).

Substrate analogues such as uracil and uracil substituted at carbon 5 with a hydroxyl-, amino- or sulfhydryl group were found to promote decarboxylation of 2-oxoglutarate in reactions catalyzed by thymine 7-hydroxylase. Since these inhibitors lacked the carbon that is normally hydroxylated it was assumed that the decarboxylation of 2-oxoglutarate observed in their presence was uncoupled from hydroxylation (33) although the pyrimidines were not analyzed after the reaction. In **paper IV** we established with 5-fluorouracil that 2-oxoglutarate de-

carboxylation was not coupled to hydroxylation of this substrate. That uracil is not hydroxylated in the reaction has been shown by Hsu et al. (31).

There is an absolute requirement for the presence of a substrate analogue for uncoupling catalyzed by thymine 7-hydroxylase. For prolyl 4-hydroxylase a great enhancement is found in the presence of substrate analogues. If there is an uncoupling without any substrate analogues is hard to evaluate since there is always protein present in the reaction mixture. Uncoupling in the absence of substrate analogues has been observed with substrate quantities of enzyme and there is a possibility that the enzyme itself could act as a substrate analogue. The increased uncoupling observed with prolyl 4-hydroxylase purified by affinity chromatograph could be due to difficulties in removing the poly L-proline from the protein (94). In our view the pseudosubstrate is needed to accomplish a conformational change of the enzyme to enable oxygen to bind, whereafter the oxygen, complexed with iron, could react with 2-oxoglutarate.

The steady state kinetics of the uncoupled reaction also show that the ordered addition of substrate is retained in the partial reaction (paper IV). An important difference between the partial and complete reaction is an increased need for ascorbate in the partial reaction. This has been observed not only with 5-fluorouracil but also with other pyrimidines assumed to be uncouplers (33). With 5-mercaptouracil the ascorbate requirement is similar to that in the complete reactions and sulfoxidation of this substrate may be a possibility. In the complete reaction both oxygen atoms are incorporated into organic substrates and no external reductant is formally needed. If monopersuccinic acid were the final product in the partial reaction, the enzyme form left after the partial reaction would be the same as in the complete reaction. As this is not the case a more likely pathway is one where free monopersuccinic acid is never formed and the oxygen — oxygen bond is broken either after, or synchronously with, decarboxylation of the peroxyacid intermediate when still ligated to ferrous ion. One oxygen atom would thus be retained in an oxo-iron complex. The uncoupled reaction shows that 2-oxoglutarate does react with oxygen without any changes of the pseudosubstrate, it also indicates that an oxidized enzyme form is generated. If this enzyme form is an intermediate in the reaction or a product of a side reaction must await further experimentation.

Tritium isotope effects

In a *ter* reactant ordered mechanism no intermolecular tritium isotope effect is expected at saturating concentrations of the substrate which binds after the labeled substrate (96,97,98,99,100). When the concentration of this substrate is lowered it should be possible to detect an isotope effect if there is no irreversible step before the isotope-sensitive step.

The reaction mechanism proposed for thymine 7-hydroxylase implies an irreversible reaction between 2-oxoglutarate and oxygen before the bond-breaking step takes place. To rule out the existence of a tritium isotope effect the experiments have to be carried out at reduced oxygen tension to avoid suppression of the isotope effect due to oxygen saturation of the enzyme.

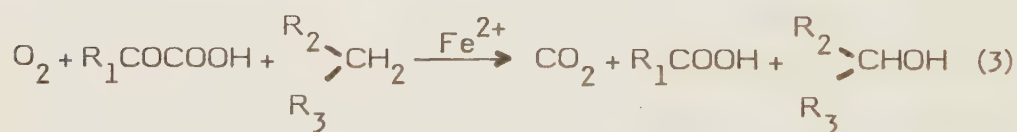
Thymine is hydroxylated in the methyl group which is torsio-symmetric. This means that both intra- and intermolecular isotope effects have to be considered when tritium tracer is used. This complicates the determination of a possible intermolecular isotope effect, but offers an unique opportunity to get a good estimate of the intrinsic isotope effect. The intramolecular isotope effect could be subject to suppression due to commitment factors as discussed by Miwa *et al.* (101). The suppression, however, is likely to be small in this case especially as the isotope competition is between rapidly equilibrating positions in the methyl group and the reversibility of the isotope sensitive step is likely to be small. An estimate of the intrinsic isotope effect is of value since it reflects the events at one single step in the enzyme reaction. The magnitude of this effect could give information on the chemical nature of that particular step.

In **paper V** intra- and intermolecular isotope effects in the reaction catalyzed by thymine 7-hydroxylase have been determined after analysis of the different influences of these effects on the specific activity ratios obtained in substrate and products at different degrees of tritium substitution of the substrate. The existence of an intermolecular isotope effect has been excluded in experiments with tritiated thymine performed at an oxygen concentration well below the K_m for oxygen. k_H/k_T of the intramolecular isotope effect has been estimated to be about 6.5 which means a k_H/k_D of 3.7 if the Swain relationship (102) is used. This is not strictly correct since the intramolecular isotope effect could differ from the intrinsic isotope effect but the error introduced by use of this relationship is probably small.

No detailed investigation of isotope effects in other 2-oxoglutarate dependent dioxygenases has been reported. However, tritium-release assays have been developed for several of these enzymes (103,104,105) and generally no isotope effects have been found. One exception is the mammalian γ -butyrobetaine hydroxylase, where both enrichment of isotope in the substrate (106) and measurements of tritium release to water (107) is indicative of a substantial intermolecular isotope effect. This divergent property of γ -butyrobetaine hydroxylase can not be explained by differences in the steady state kinetics. The proneness of this enzyme to uncouple the 2-oxoglutarate decarboxylation from hydroxylation, raises the possibility that isotope substitution leads to uncoupling, which is currently under investigation in our laboratory. Uncoupling due to isotope substitution could also explain the very high value reported for isotope enrichment in unreacted γ -butyrobetaine (106) which by far exceeds the theoretically possible value.

MECHANISM - THEORETICAL CONSIDERATIONS

The 2-oxoglutarate-dependent oxygenases are ferrous enzymes which insert oxygen into inactive carbon hydrogen bonds in a reaction coupled with oxidative decarboxylation of 2-oxoglutarate according to eq 3.



Observations relevant to the mechanistic considerations are:

The substrates contain a carbonyl or a hydroxyl group near the position to be hydroxylated.

2-Oxoglutarate decarboxylation can be uncoupled from hydroxylation.

There are indications of a thiol group in the active centre of the enzymes.

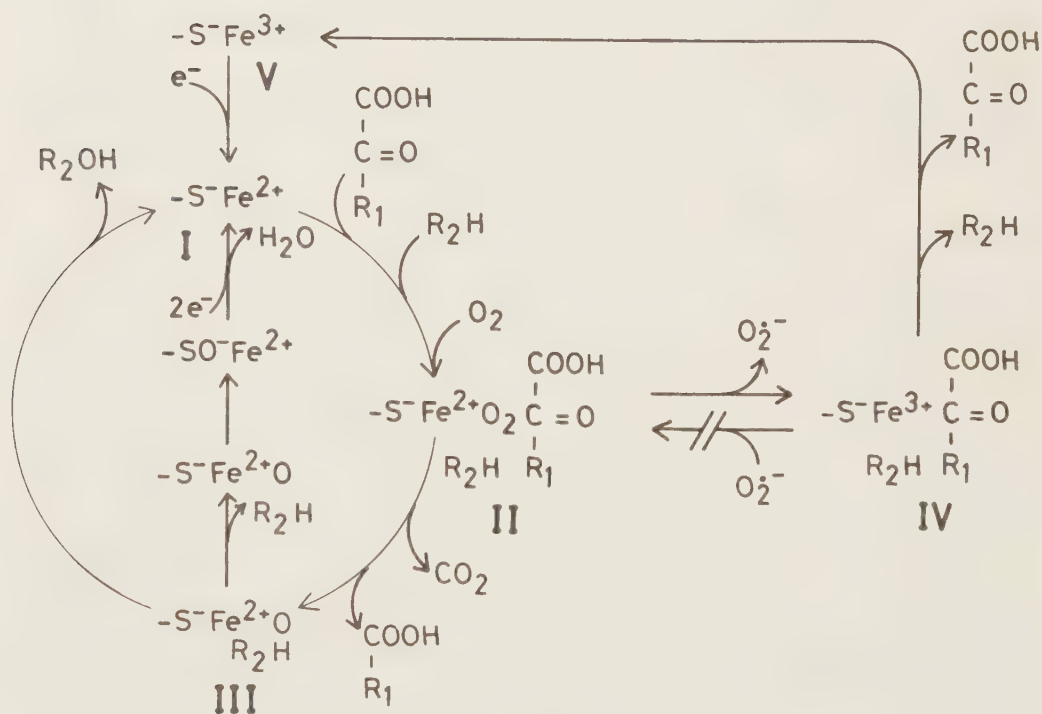
Two different types of oxidative inactivation have been observed.

Both organic substrates are bound to the enzyme before the reaction with oxygen starts.

Generally no intermolecular tritium isotope effects seem to occur.

The magnitude of the isotope effect in the bond-breaking step has been estimated to $\frac{k_H}{k_D} = 3.7$ for thymine 7-hydroxylase.

These facts together with analogies drawn from other hydroxylation reactions both enzymatic and nonenzymatic is the basis for our present opinion of the reaction mechanism of the 2-oxoglutarate-dependent oxygenases presented in **Scheme IV**.



SCHEME IV

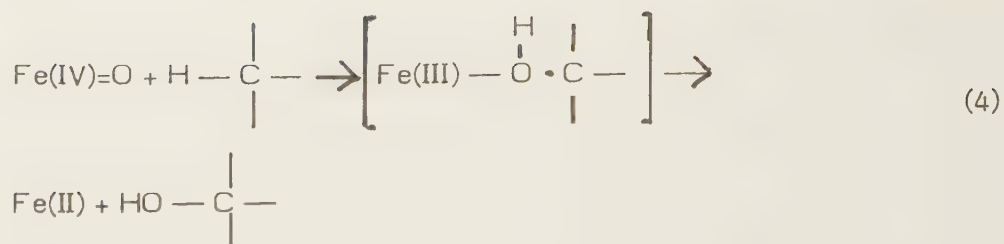
The active enzyme (I) contains Fe^{2+} which might be ligated to a thiolate. The Fe^{2+} is in equilibrium with Fe^{2+} in the solution. 2-Oxoglutarate is first added to this enzyme form, followed by the substrate to be hydroxylated. This order of addition was established in **paper III** and further substantiated in **paper IV** for thymine 7-hydroxylase. This order of addition of substrates *i.e.* that binding of the substrate to be hydroxylated precedes the binding of oxygen is a general principle found in iron-containing oxygenases (77,90). O_2 is then added to the ferrous ion to form complex II.

As discussed by Siegel (108) different iron-oxygen complexes are possible which all would be reasonably reactive intermediates able to accomplish oxidation at the carbonyl carbon of 2-oxoglutarate. Since oxidative inactivation during turnover conditions is likely to occur at this stage, a superoxide complex is possible, from which superoxide could dissociate to form the inactive complex **IV**. Myllylä *et al.* (109) have proposed the involvement of superoxide anion based on inhibition studies with low molecular copper chelates, but as has been shown from our laboratory (110) the inhibition observed with several superoxide scavengers is complex in nature and other reasons than mere superoxide anion scavenging could account for the effects observed. What is quite clear, however, is that superoxide in solution is of no importance for the enzyme reaction, and that superoxide-generating systems cannot substitute for ascorbate. In the main reaction the $\text{Fe}^{2+}-\text{O}_2$ complex reacts with 2-oxoglutarate to form a peroxide. Monoperoxysuccinic acid is probably not formed as a discrete intermediate, but the oxygen-oxygen bond is probably broken at this stage in a heterolytic cleavage, and a complex with Fe^{2+} and an oxygen atom is formed (**III**). The reason to abandon the original mechanism of Hamilton (36), who proposed direct transfer of oxygen from monoperoxysuccinic acid to the substrate, is that normal peroxy acids, such as monopersuccinic acid, require long reaction times at elevated temperature to hydroxylate aliphatic carbons, whereas iron-associated oxidants derived from peroxy acids can effect such hydroxylations much more easily (111). When succinate is formed in an uncoupled reaction the enzyme is left in an oxidized form, which would not have been the case if the peracid was the product of this reaction (**paper IV**). The iron-oxo complex in **III** has been written as a Fe^{2+} oxygen atom complex; the formal oxidative state of iron in this complex is however Fe(IV). The idea of formation of a highly oxidized iron-oxo intermediate in the 2-oxoglutarate-dependent oxygenase reactions has been discussed by Siegel (108). He also stresses that the exothermic decarboxylation of the peroxy intermediate would be the driving force required to produce the active iron oxidant. Energy transfer does not, however, seem to be necessary as judged from nonenzymatic reactions. Groves and Mc Clusky (112) have described a hydroxylation of cyclohexanol in acetonitrile by a ferrous ion - peroxy-acid system. This reaction has been found to be stereoselective and the hydroxyl is formed from peracid - oxygen without equilibration with water. Formation of an iron-oxo intermediate through heterolysis of the

peroxide has been envisaged. Stereoselectivity is obtained by ligation of the hydroxyl of cyclohexanol to the iron. Although the precise nature of the iron-associated oxidant is not clear, Fe(IV)=O would be the complex of choice, as recently discussed in a review by Groves (111). As has been pointed out the substrates of the 2-oxoglutarate-dependent oxygenases have carbonyls or hydroxyls that could be ligated to iron and thereby direct the oxygen transfer. For thymine 7-hydroxylase it has been shown that the carbonyl at carbon 4 is essential for hydroxylation (63). The $k_{\text{H}}/k_{\text{D}}$ of 3.2 found for the cyclohexanol hydroxylation (112) is another finding that indicates a relation between this model reaction and the 2-oxoglutarate-dependent oxygenases. The likely formation of a peroxide in the 2-oxoglutarate-dependent oxygenases makes analogies drawn from peroxidase chemistry not that far remote. The heme iron of these enzymes has long been known to exist at high oxidation states and the so called compound I has been identified as an Fe(V)=O or Fe(IV)=O with one electron localized on the porphyrin (113,114).

The formation of a reactive iron-oxo complex has also been suggested, in reactions catalyzed by cytochrome P-450 (Reviewed in 90,115,116). Although these enzymes have been extensively studied by spectroscopy the nature of the active hydroxylating agent is not settled. The fifth ligand of iron in these enzymes is thiolate, which would contribute to the very electron-rich environment of the iron. Until recently a heterolytic cleavage of the oxygen—oxygen bond has been favored in these reactions in analogy with the mechanism for peroxidases and this has not been contradicted for oxygenase reactions of cytochrome P-450 enzymes. However, a homolytic cleavage has been suggested for the oxygen—oxygen bond cleavage (117,118,119), as evidence has accumulated that in the peroxide-dependent hydroxylation catalyzed by these enzymes a carboxy radical intermediate is formed and that this reaction could initiate radical chain reactions. The peroxide dependent reaction is suicidal in nature, that is the enzyme is rapidly destroyed when catalyzing this reaction, which indicates that the reaction intermediates of this reaction are different from those in the normal oxygenase reaction.

There is no indication of formation of a carboxy radical in the reaction catalyzed by thymine 7-hydroxylase as discussed in **paper IV**. We thus consider the Fe(IV)=O complex as the most likely in this reaction. As discussed by Groves (112) the actual hydroxylation step would then proceed according to eq. 4.



When 2-oxoglutarate decarboxylation is uncoupled from hydroxylation the enzyme is left in an oxidized state (VI). The possibility of oxidative side reactions of this complex has been illustrated with the formation of a sulfoxide, but formation for instance of a S-S bridge is another possibility. Reductive activation of this enzyme form or prevention of its formation is accomplished by high concentrations of ascorbate in the reaction of thymine 7-hydroxylase (**paper IV**), whereas dithiothreitol is needed to reduce the enzyme form produced from oxidative inactivation when prolyl 4-hydroxylase is incubated with 2-oxoglutarate and oxygen (80).

ACKNOWLEDGEMENT

I want to express my deep gratitude to

- Professor Sven Lindstedt, who introduced me into the field of biochemistry, for much valuable advice and many stimulating discussions, and for his encouragement throughout this work.
- Professor Göran Lindstedt for fruitful discussions and valuable advice.
- Marianne Lindblad, who was a patient teacher in the laboratory, and for stimulating collaboration.
- Louise Hellner for stimulating collaboration.
- Inger Andersson for skilful technical assistance and her interest in the work.
- Anne-Marie Fredholm, for her support during the completion of this work, and for elaborate and skilful assistance with the lay-out and typing of the manuscript.
- Anna-Greta Janson, for careful and skilful typing of the manuscript.
- My colleagues for stimulating discussions and valuable help and all other members of the staff for always being kind and helpful

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